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FULL TEXT IS AVAILABLE IN THE ALL FORMAT
AB The use of peptides and proteins in medical treatments has become popular as a result of advances in biotechnology. However, their complicated structures make these substances highly susceptible to degradation. This article reviews the basic structures of peptides and proteins, the causes and mechanisms of their degradation, and some possible approaches for improving their stability.

homologous HIV-2 challenge was obtained in one out of four animals that received the low-serum dose and in two of three animals that received the higher serum dose (Table 2). All seven controls as well as 27 monkeys in separate experiments became infected with the same HIV-2 challenge dose.

In a second experiment, four cynomolgus monkeys were given 9 ml kg^{-1} anti-SIV$_{sm}$ serum and 6 h later challenged with 10-100 MID$_{50}$ of SIV$_{sm}$. Two control animals received 20 ml normal monkey serum before challenge. Three out of four monkeys pretreated with SIV$_{sm}$ antiserum showed no signs of infection following a homologous SIV$_{sm}$ challenge, whereas the two controls became infected (Table 3). In another experiment (not shown), nine cynomolgus monkeys were inoculated with the same dose, 10-100 MID$_{50}$ of SIV$_{sm}$, and all nine monkeys became infected.

Antibody titres as determined with whole-antigen enzyme-linked immunosorbent assay (ELISA) declined after challenge with a half-life of approximately 7-9 days and became undetectable at 3-4 months in protected animals (monkeys B22, B52, B6 and B31-33). This further indicates that active infection did not occur during 6-10 months of follow-up after live virus challenge. Five out of seven monkeys treated with 9 ml kg^{-1} of anti-HIV-2 or anti-SIV serum resisted the homologous cell-free virus challenge, whereas a lower dose of anti-serum appeared to be less efficient. Our data suggest that the titre of viral specific antibodies may be critical for protection, but the prechallenge antibody titres in individual monkeys did not show a clear correlation with protection. Larger studies will be required to establish both the amount and quality of antibodies that give protection.

Successful passive immunization against primate lentiviruses in a nonhuman primate has to our knowledge not been reported previously. Prince *et al.*[11] reported that passive transfer of pooled IgG from HIV-1-infected humans failed to protect chimpanzees against live HIV-1 virus challenge. But passive transfer of anti-envelope antibodies has been shown to protect against murine and feline oncogenic retroviruses[12,13] and recently Kataoka *et al.* showed that human antibodies to human T-cell leukaemia virus type I (HTLV-I) prevented HTLV-I infection in rabbits[14]. We have shown here that antibodies alone are sufficient to protect against HIV-2 and SIV infection in a nonhuman primate. Studies on post-exposure prophylaxis and protection against heterologous virus strains are in progress. □

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Fibronectin and VLA-4 in haematopoietic stem cell–microenvironment interactions

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THE self-renewal and differentiation of haematopoietic stem cells occurs *in vivo* and *in vitro* in direct contact with cells making up the haematopoietic microenvironment[1-4]. In this study we used adhesive ligands and blocking antibodies to identify stromal cell-derived extracellular matrix proteins involved in promoting attachment of murine haematopoietic stem cells. Here we report that day-12 colony-forming-unit spleen (CFU-S$_{12}$)[5] cells and reconstituting haematopoietic stem cells attach to the C-terminal, heparin-binding fragment of fibronectin by recognizing the CS-1 peptide of the alternatively spliced non-type III connecting segment (IIICS) of human plasma fibronectin. Furthermore, CFU-S$_{12}$ stem cells express the α_4 subunit of the VLA-4 integrin receptor, which is known to be a receptor for the CS-1 sequence, and monoclonal antibodies against the integrin α_4 subunit of VLA-4 block adhesion of CFU-S$_{12}$ stem cells to plates coated with the C-terminal fibronectin fragment. Finally, polyclonal antibodies against the integrin β_1 subunit of VLA-4 inhibit the formation of CFU-S$_{12}$-derived spleen colonies and medullary haematopoiesis *in vivo* following intravenous infusion of antibody-treated bone marrow cells.

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Our role of stromal cells making up the haematopoietic microenvironment in the maintenance of haematopoiesis may involve secretion of extracellular matrix (ECM) proteins to provide anchorage sites for colocalization of stem cells and growth factors[6-8]. To examine the role of ECM in haematopoietic stem-cell adhesion, we first analysed the binding of CFU-S$_{12}$ by adherence depletion assays in which a limiting dilution of freshly isolated bone marrow cells was incubated on ECM produced by a murine bone marrow-derived stromal cell line, U2, capable of replacing the haematopoietic microenvironment of a long-term marrow culture in supporting haematopoiesis *in vitro*[9,10]. After a 2-h incubation at 37 °C, the nonadherent population of cells was collected and assayed for CFU-S$_{12}$. Incubation of bone-marrow cells on U2 ECM resulted in the depletion of 50% of CFU-S$_{12}$ compared with cells incubated on gelatin-coated dishes (control) (11.7 ± 1.3 versus 24.3 ± 3.1 nonadherent CFU-S$_{12}$; Table 1). To determine the long-term haematopoietic reconstituting capacity of U2 ECM-bound cells, a larger inoculum of bone marrow cells obtained from male mice was incubated on the U2 ECM. After careful removal of nonadherent cells, the bone marrow cells adherent to U2 ECM were collected and injected into lethally irradiated female mice. Southern blot analysis of DNA isolated from thymus, bone marrow and spleen cells from these female mice 4 months post-transplant revealed a prominent Y chromosome-specific hybridizing band (Fig. 1*a*), demonstrating that cells adhering to U2 ECM not only contain CFU-S$_{12}$, but are also capable of multilineage and long-term

reconstitution of irradiated animals.

Although stromal cell ECM (including U2) is composed of collagens, laminin, and fibronectin[9,11], we found that neither collagen types I, III and IV, nor laminin promoted significant attachment of CFU-S_{12} (data not shown). As primitive erythroid progenitors (K. Goltry and V. Patel, manuscript in preparation) and lymphoid cells[12-14] attach specifically to the C-terminal, heparin-binding fragments of fibronectin, we analysed stem cell binding to dishes coated with intact fibronectin or its chymotryptic fragments. Incubation of bone marrow cells on bacteriological plates coated with a C-terminal heparin-binding fragment of fibronectin (HBD) of relative molecular mass 30,000–35,000 (M_r 30-35K) resulted in 55% reduction in the number of nonadherent CFU-S_{12} (Table 1). As in previous studies of stem cells[15] and primitive BFU-E (K. Goltry and V. Patel, manuscript in preparation), no adherence of CFU-S_{12} was demonstrated on intact fibronectin (Table 1). Furthermore, no appreciable adherence of CFU-S_{12} was detected on a 115K cell-binding domain containing the RGDS sequence (Table 1), or a 42K C-terminal fibronectin fragment containing the HBD, but not a second cell-binding domain located in this same region of fibronectin called the CS-1 sequence (data not shown). The lack of adherence to the 42K fragment was in agreement with our findings that preincubation of U2 ECM with heparin sulphate had minimal effects on CFU-S_{12} adherence (Table 1). These data suggest little, if any, adhesion of CFU-S_{12} to the heparin-binding site (II) (ref. 16) located in both the 42K and the 30-35K fragments and implicate the CS-1 sequence[17-19] in this adherence.

This possibility was examined by measuring adhesion of CFU-S_{12} to bacteriological plates coated with synthetic CS-1 peptide covalently crosslinked to bovine serum albumin (BSA). Incubation of bone marrow cells on CS-1/BSA resulted in 50% reduction of nonadherent CFU-S_{12} compared to bone marrow cells incubated on plates coated with BSA alone or CS-3/BSA conjugate (control synthetic peptide present in IIICS amino-acid sequence) (Table 1). The presence of CS-1 sequence in the 35K fragment of fibronectin was confirmed by immunoblotting with anti-CS-1 peptide antibody, and the presence of CS-1 containing differentially spliced messenger RNA in U2 stromal cells was confirmed by northern blot analysis using a synthetic 75-base oligonucleotide probe representing the CS-1 sequence of rat fibronectin (ref. 20; R. S. Dwivedi, V.P.P. and D.A.W., unpublished data). Adherence of CFU-S_{12} to the 30-35K fibronectin fragment was dose-dependent and saturable (data not shown). CFU-S_{12} that were nonadherent to optimal concentrations of 30-35K fragment gave rise to fewer multilineage spleen colonies (1 out of 15 colonies, or 6.7%) when compared with control injections (12 out of 28 colonies, or 42.9%), demonstrating that the adherence to this fragment defines a more primitive CFU-S compartment. Haematopoietic stem cells (from male mice) adherent to the 30-35K domain were also capable of multilineage haematopoietic reconstitution of irradiated female mice (Fig. 1b), demonstrating that cells adherent to this fragment include long-term reconstituting stem cells.

Cell adhesion to ECM proteins is mediated by the integrin family of cell-surface receptors[21]. Adhesion of some lymphoid cells to the CS-1 sequence of fibronectin is mediated by the VLA-4 integrin, a heterodimer consisting of α_4 and β_1 subunits[17,18] and this α_4-subunit is apparently shared by the

TABLE 1 Analysis of CFU-S adherence to U2 stromal cell ECM and cell adhesion domains of fibronectin

Substrates	Nonadherent CFU-S	Depletion (%)
Control	24.3 ± 3.1*	0
ECM	12.7 ± 1.3	50
ECM + heparin†	8.5 ± 2.5	65
Control	40.5 ± 2.9*	0
Fibronectin	41.0 ± 1.3	0
HBD (30-35K)	18.0 ± 8.4	55
CBD (115K)	38.0 ± 4.7	0
Control	8.0 ± 2.6‡	0
CS-1/BSA	4.0 ± 3.5	50
CS-3/BSA	7.8 ± 2.7	0
Control	6.7 ± 1.3§	0
HBD (30-35K)	2.7 ± 1.3	60
HBD (30-35K) + αH_2Ab	3.4 ± 2.4	49
HBD (30-35K) + LPAM-1 Ab	7.0 ± 1.1	0

Results shown are representative experiments. The data are expressed as mean ± s.d. of at least four animals per experiment. Bone marrow cells were collected as described[10] from male C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Maine). Extracellular matrix was prepared as previously described[12] after growing U2 cells on tissue culture plates (either 6-well or 10-cm; Corning, New York) to confluence. In some experiments, ECM was treated for 30 min with 500 μg heparin sulphate (Sigma) in phosphate-buffered saline (PBS) per 60-mm dish. Plates were then washed free of unbound heparin with PBS before addition of cells. For CFU-S_{12} adherence, limiting dilutions (1.3×10^5) of plastic adherence-depleted bone marrow cells were then placed on U2, U2 ECM (plates coated with 0.1% gelatin (Sigma) were controls for nonspecific binding of CFU-S_{12} in the ECM-binding experiments) or bacteriologic plates coated with protein fragments (see below). For attachment assays to fibronectin fragments, 35-mm bacteriologic petri dishes (Falcon, Lincoln Park, New Jersey) were coated with 2 ml PBS solutions containing 20 μg ml^{-1} indicated substrates or 2% BSA (control), as previously described[33]. For attachment assays to synthetic peptides, 24-well nontissue culture plates (Costar, Cambridge, Massachusetts) which had been coated with 250 μg per well BSA or BSA conjugate (50-60 mol peptide per mol BSA) were used. After a 2-h incubation at 37 °C in 5% CO_2, nonadherent cells were collected and saved. For blocking studies, bone marrow cells (2.5×10^5 ml^{-1}) were preincubated at 4 °C with 16 μg ml^{-1} of LPAM-1 (or rat anti-H2 pan monoclonal antibodies M1/42.3.9.8.HLK, 1:100 dilution)[34] for 30 min rotating end-over-end. Antibody-treated cells (1.25×10^5) were plated in 0.5 ml at 4 °C in 24-well bacteriological plates which had been precoated with 30-35K fibronectin fragment (or 2% BSA) as above. After a 2-h incubation, nonadherent cells were carefully collected, trypsinized for 1 min at 37 °C to remove remaining antibody. All nonadherent cells were collected by three gentle washes with warmed media containing 2% BSA and all washes were combined and injected into lethally irradiated mice (1,250 rads, split dose with 3 h between doses at 145 rads per min; this dose of irradiation is lethal to 100% of mice not receiving bone marrow infusions). Spleens collected 12–14 days post-transplant were fixed and surface colonies counted as described[10].

* Nonadherent CFU-S after overlaying 3×10^5 cells for 2 h on ECM or protein-coated plates. Control was 0.1% gelatin or 2% BSA-coated plates.

† ECM was preincubated with 500 μg heparin sulphate per 60-mm dish before incubation of cells, see legend.

‡ Nonadherent CFU-S after overlaying 9×10^4 cells for 2 h on peptide-coated plates. Control, BSA-coated plates.

§ Nonadherent CFU-S after overlaying 1.25×10^5 antibody-treated cells for 2 h on peptide-coated plates. Control, BSA-coated plates.

TABLE 2 Expression of VLA-4 on CFU-S

Primary antibody	Number of cells injected	Spleen colonies	CFU-S content
Preserf	$2 \cdot 10^5$	10.3 ± 0.8	5.0 per 10^5
LPAM-1	$0.9 \cdot 10^5$	6.2 ± 2.2	6.9 per 10^5
Mac-1	$2 \cdot 10^5$	0	<0.5 per 10^5

Plastic adherence-depleted cells and low-density bone marrow mononuclear cells isolated by centrifugation on a Ficoll-hypaque gradient (Histopaque-1119, Sigma) (10^6 ml^{-1}) were incubated with undiluted antibodies to VLA-α_4 (murine anti-α_4 monoclonal PAM-1[22], 1:50 dilution of rat anti-murine Mac-1 monoclonal antibody[23] (Boehringer Mannheim) or in a minimum essential medium (MEM) (Gibco, Grand Island, New York) containing 5% fetal calf serum alone at 4 °C for 45 min while rotating end over end. Cells were then washed twice with ice-cold PBS and treated with a 1:100 dilution of FITC-labelled goat anti-rat or goat anti-mouse IgG (Tago, Burlingame, California) for 45 min at 4 °C. After incubation with the secondary antibody the cells were washed twice with ice cold PBS and resuspended in α MEM containing 5% fetal calf serum at $1-3 \times 10^6$ cells per ml for cell sorting. Fluorescein-labelled cells were sorted on a Cytofluorograf IIs (Ortho Diagnostics) containing an Argon laser and band filter for 510–540 nM wavelengths using gates selected for medium cell size (8–10 μm) based on forward and 90° light scatter. Positive cells were selected on the basis of fluorescence intensity that was >2 s.d. above the mean channel of negative control cells (unstained by first antibody) and 36% of positive cells exhibiting medium bright fluorescence were collected on ice, washed once with media containing 5% fetal calf serum, trypsinized for 2 min at 37 °C, washed twice in ice-cold medium containing 5% fetal calf serum, and injected into lethally irradiated syngeneic recipients. Data presented were obtained from one of two independent experiments showing similar results.

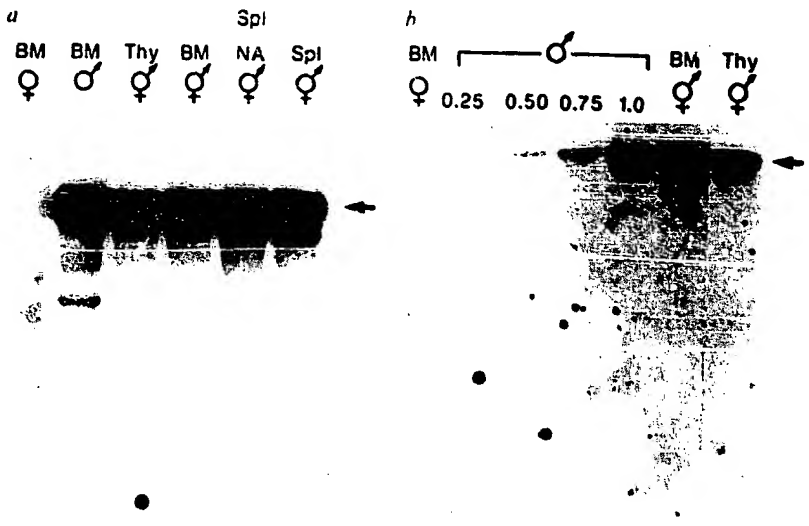

FIG. 1 Southern blot analysis of female mice transplanted with male bone marrow cells adherent to U2 ECM (*a*) or 30–35K (*b*) chymotryptic fragment of fibronectin. For reconstitution assays on ECM (*a*) (3×10^6) bone marrow cells depleted of plastic adherent cells were plated onto 10 cm tissue culture dishes (Corning) containing cell-free U2 ECM and allowed to adhere for 2 h. The adherent cells were then injected into lethally irradiated female mice (C3H/HeJ, Jackson Laboratories). Four months after transplantation, DNA was prepared from thymus, spleen and bone marrow cells, cut with *Bam*HI, size fractionated on a 1% agarose gel, transferred to a nylon filter (Magnagraph, Micron Separations, Westboro, Massachusetts) and probed with ^{32}P-labelled Y chromosome-specific probe, PY-2 (ref. 34). Lanes are labelled with the source of cells used for the DNA preparation; BM♀, female bone marrow (negative control); BM♂, male bone marrow (positive control); Thy♂, thymus; BM♂, bone marrow; Spl NA♂, non-adherent spleen cells; Spl♂, total spleen cells all from a female mouse transplanted with male cells adherent to U2 ECM. Arrow, major male-hybridizing band running at 14 kilobases. The figure shows tissues of one representative mouse of a group of three. For reconstitution assays on fibronectin fragments (*b*), bone-marrow cells were plated onto 30-mm bacteriological dishes (Falcon) coated with 40 μg per dish of 30–35K chymotryptic fragment of fibronectin (containing CS-1) and adherent cells collected and transplanted, as described above. Four months after transplant, tissues were collected from mice, DNA prepared and Southern blots performed as in *a* except additional controls for copy number were included by preparing DNA from mixtures of male and female bone marrow to represent 25, 50 and 75% male cells. Results from a representative transplant recipient of a group of three mice.

Peyers' patch-specific lymphocyte homing receptor[22]. We used a monoclonal antibody to murine α_4, LPAM-1 (ref. 22) and fluorescence-activated cell sorting (FACS), to determine whether CFU-S_{12} express this subunit of VLA-4. About 36% of bone marrow cells stain positive with LPAM-1 (data not shown). Injection of VLA-4-positive bone marrow cells (collected from a gate representing the middle 33% of fluorescence intensity) gave rise to CFU-S_{12}-derived spleen colonies in lethally irradiated mice (Table 2). By contrast, bone marrow sorted for the presence of Mac-1 antigen[23], which is expressed on more differentiated myeloid cells, failed to form spleen colonies when injected at concentrations 2–5 times higher than LPAM-1-positive cells (Table 2). The expression of the α_4 subunit was further shown to be functionally important as incubation of bone marrow cells with LPAM-1 antibody blocked attachment to 30–35K fibronectin-fragment-coated dishes (Table 1).

Consistent with this result are our observations on the effect of rabbit polyclonal antibodies against the common β_1-subunit of the VLA integrin receptors on the lodgement of injected stem cells in the spleen and bone marrow *in vivo*. Preincubation of limiting dilution of bone-marrow cells with intact anti-FnR (β_1)[14] immune IgG or anti-FnR F(ab)$_2$ fragments resulted in significant reduction in the ability of marrow cells subsequently to give rise to spleen colonies (Fig. 2) or myeloid colonies in the femur (data not shown), whereas control incubations performed in the presence of either preimmune IgG or anti-pan H2 (ref. 24) monoclonal antibody was without significant effect (Fig. 2).

We conclude that the adhesion of primary haematopoietic stem cells to stromal cell ECM is partly promoted by the proteolytic fragments of fibronectin containing the alternatively spliced region of the IIICS domain and we suggest that this interaction is likely to be mediated by the integrin receptor VLA-4 (α_4/β_1). As only one out of three potential products of the alternative splicing of the fibronectin pre-mRNA has been shown to contain CS-1 sequence in the rat[20], it will be important to determine the role of stromal cell expression of fibronectin splicing variants and ECM protein remodelling by proteolysis in the previously observed nonrandom, spatial distribution of haematopoietic stem cells in the microenvironment[25,26]. These

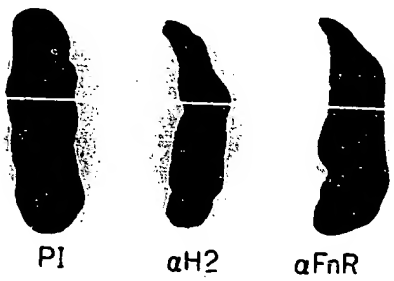

FIG. 2 Inhibition of CFU-S-derived spleen-colony formation by anti-fibronectin receptor antibody. Antibodies used included affinity-purified rabbit anti-β_1 (ref. 14) and Fab$_2$ fragment prepared by pepsin treatment followed by protein A adsorption of Fc-containing antibodies; and rat anti-H_2 monoclonal antibodies. Blocking of CFU-S homing to spleen *in vivo* was done by incubating limiting dilution bone marrow cells ($1-3 \times 10^5$ ml^{-1}) depleted of plastic adherent cells with 100 μg ml^{-1} rabbit anti-fibronectin receptor (mouse) IgG, 100 μg ml^{-1} rabbit preimmune IgG, or 1:50 dilution anti-pan H_2 (as an irrelevant antigen expressed on CFU-S) at 4 °C for 45 min while rotating end over end. After this incubation, cells were washed twice in ice-cold PBS and resuspended in ice-cold α-MEM containing 5% FCS. Cells were kept on ice until injected slowly into lethally irradiated mice. Spleens were harvested 12–14 days post-transplant and analysed as above. Figure shows representative spleens from one experiment.

interactions may have important implications in the localization of intravenously injected stem cells to the medullary cavity during bone marrow transplantation and modulation of expression of VLA-4 may have a role in loss of adhesion of leukaemic blast cells to stromal cells noted during blast crisis in chronic myelogenous leukaemia[27]. By analogy to lymphocyte homing mechanisms, stem-cell stromal interactions may use multiple ligand-receptor interactions including VLA-4/CS-1 described here and lectins described previously[28]. Finally, the recent characterization of the steel (*Sl*) gene product as a transmembrane growth factor strengthens the possibility that an important role of the haematopoietic microenvironment is to provide anchorage sites for stem cells to promote local interactions with membrane-bound cytokines[29-31]. □

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A recycling pathway between the endoplasmic reticulum and the Golgi apparatus for retention of unassembled MHC class I molecules

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ASSEMBLY of class I major histocompatibility complex (MHC) molecules involves the interaction of two distinct polypeptides (the heavy and light chains) with peptide antigen. Cell lines synthesizing both chains but expressing low levels of MHC class I molecules on their surface as a result of a failure in assembly and transport have been identified[1]. We now report that although the apparent steady-state distribution in these cells of class I molecules is in the endoplasmic reticulum (ER), the molecules in fact are recycled between the ER and Golgi, rather than retained in the ER. This explains the failure of class I molecules to negotiate the secretory pathway. Class I molecules do not seem to be modified by Golgi enzymes, suggesting that the proteins do not reach the Golgi apparatus during recycling. But morphological and subcellular fractionation evidence indicates that they pass through the cis Golgi or a Golgi-associated organelle, which we postulate to be the recycling organelle. This compartment, which we call the 'cis-Golgi network', would thereby be a sorting organelle that selects proteins for return to the ER.

We examined the mutant cell lines[1] and failed either to detect carbohydrate processing of class I molecules in the Golgi or to coprecipitate much light chain with an anti-heavy chain antibody (data not shown). Immunofluorescence staining of these cells with antibodies to class I was consistent with the biochemistry, revealing a typical ER distribution (Fig. 1*a*)[2], not a Golgi one as revealed by an antibody to the Golgi marker manosidase II (man II)[3] (Fig. 1*b*).

The drug brefeldin A induces the rapid redistribution of Golgi proteins into the ER along a pathway that is inhibited by microtubule-disrupting agents, energy poisons and reduced temperatures[4]. We therefore reasoned that the retrograde transport, and hence distribution, of proteins that are normally recycled might also be altered by lowering the temperature or by depolymerizing microtubules. This would enable us to distinguish whether class I molecules are truly retained in the ER or recycled in one of the mutant cell lines, CMT[1]. When CMT cells were incubated at 16 °C for 2 h and then warmed for 5 min to 37 °C, class I molecules redistributed to a Golgi-like pattern (Fig. 1*c*, *d*), whereas the distribution of the ER marker remained unchanged (not shown). Depletion of ATP with 2-deoxy-D-glucose and sodium azide, which inhibit ER to Golgi membrane traffic[6], prevented the change in distribution of class I molecules at 16 °C (not shown). The distribution of a nonresident ER protein, a truncated form of the T-cell-receptor α chain ($TCR\alpha_{GPI}$), which cannot be secreted but is retained in the ER, was similarly examined. When expressed in Chinese hamster ovary cells kept at 37 °C, $TCR\alpha_{GPI}$ is processed and distributed in the same way as retained MHC class I molecules[5]. Unlike class I, however, $TCR\alpha_{GPI}$ did not assume a Golgi-like distribution at 16 °C (Fig. 1*g*, *h*).

Redistribution of class I molecules at 37 °C can be induced using nocodazole, which causes microtubule depolymerization. Within 40 min of nocodazole treatment, class I molecules changed from having a punctate/reticular (ER-like) distribution to having a scattered distribution, corresponding to structures that lacked ER resident proteins, but which often colocalized with the Golgi marker (Fig. 1*e*, *f*). The distribution of $TCR\alpha_{GPI}$ did not change after nocodazole treatment.

Class I molecules in cells incubated at 16 °C and then rewarmed to 37 °C (in the presence of cycloheximide to prevent the introduction of newly synthesized class I molecules into the